STUDIES ON THE ORIGIN OF THE FORM Ib MAMMALIAN DNA-DEPENDENT RNA POLYMERASE

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1. Introduction

Four forms of animal cell DNA-dependent RNA-polymerase can be extracted from nuclei and separated [1–3]. Forms Ia and Ib are localized in the nucleolus [2] whilst forms II and III are found in the nucleoplasm [4]. We can only detect trace amounts of form III in adult rat liver. α-Amanitin inhibits form II but has no effect on the other three polymerases [1, 3]. Two methods of polymerase extraction are in current use: a low salt method in which the enzymes are slowly leached out of nuclei [1, 2]; and a high salt method in which nuclei are lysed and sonicated [2, 3, 5]. The low salt method selectively extracts the form I polymerases whilst high salt extraction liberates all forms of the enzyme.

We have reported that during low salt extraction the level of form Ib is substantially enhanced, possibly by interconversion from other polymerase forms [2]. It was suggested in a speculative model of the nuclear transcription system that form Ib results from the dissociation of form II. This paper describes two experiments designed to test whether or not interconversion between form I and II polymerases can be detected during low salt incubation of nuclei. The results do not lend support to the idea. They indicate that form Ib is derived from form Ia or a previously inactive source. However, the possibility of interconversion still cannot be ruled out.

2. Methods

2.1. *Low salt extraction*

Rat liver nuclei, prepared as by Widnell and Tata [6] with reported modifications [1], were suspended in low salt extraction buffer (0.01 M tris-HCl, pH 8, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol containing 0.07 M KCl), 0.5 ml for each 1 g of liver used, and incubated at 37° with shaking. Nuclei were spun off at 1000 *g* for 10 min and the extract dialysed overnight against the above buffer containing 0.05 M KCl, 10% glycerol and 15 mM β-mercaptoethanol. RNA polymerase assays, DEAE-cellulose and phosphocellulose chromatography were performed as before [1, 2].

2.2. *High salt extraction*

Nuclei were suspended in the low salt extraction buffer as above and, per 10 ml suspension, 10 ml 2.4 M sucrose, 0.24 ml M tris-HCl, pH 8, 0.12 ml 1 M $MgCl_2$, and 0.03 ml 1 M dithiothreitol were added. After mixing, 1.9 ml 4 M $(NH_4)_2SO_4$ was also added.

Table 1
Selective inhibition of intranuclear form II RNA polymerase by α-amanitin.

Assay conditions	Intranuclear RNA polymerase activity (cpm)	
	Form I	Form II
Plus α-amanitin	2467	1351
Minus α-amanitin	2921	9177

Nuclear suspensions (0.1 ml) were assayed for 10 minutes at 37° in the presence or absence of 2 μg/ml α-amanitin with 0.15 M KCl and 6 mM $MgCl_2$ (optimal for form I polymerases) or with 10% saturated $(NH_4)_2SO_4$ and 4 mM $MnCl_2$ (optimal for form II polymerase) as described previously [1, 2].

Nuclei were lysed by shaking gently and 12 ml portions of the mixture were sonicated at 0° for 1 min in 10 sec bursts at full power with a Branson Sonicator using a medium probe. Separation of forms Ia, Ib and II from the sonicate was carried out as reported previously [2].

3. Experimental and results

3.1. *Effect of α-amanitin on the extraction of form I polymerases from nuclei*

Low salt extraction releases form I polymerases from suspended nuclei at a linear rate for up to 60 min [1]. Most of the form Ib released is generated during the extraction from an unknown source [2]. We have suggested that since intranuclear form II activity decreases rapidly as Ib is formed Ib might be derived from form II [2]. As shown in table 1, 85% of the intranuclear activity detectable with a high salt assay using Mn^{2+} ions, which is optimal for form II, was inhibited by α-amanitin. The activity exhibited in a low salt assay using Mg^{2+} ions, which is optimal for form I enzymes, was only depressed by 15% by the drug. This shows that α-amanitin penetrates the nucleus and inhibits form II in situ.

Having demonstrated this, the effect of α-amanitin on the extraction of form I polymerases was determined. If form Ib, which makes up over 50% of the form I extracted [1, 2], is derived from form II, the drug might be expected to significantly reduce the amount

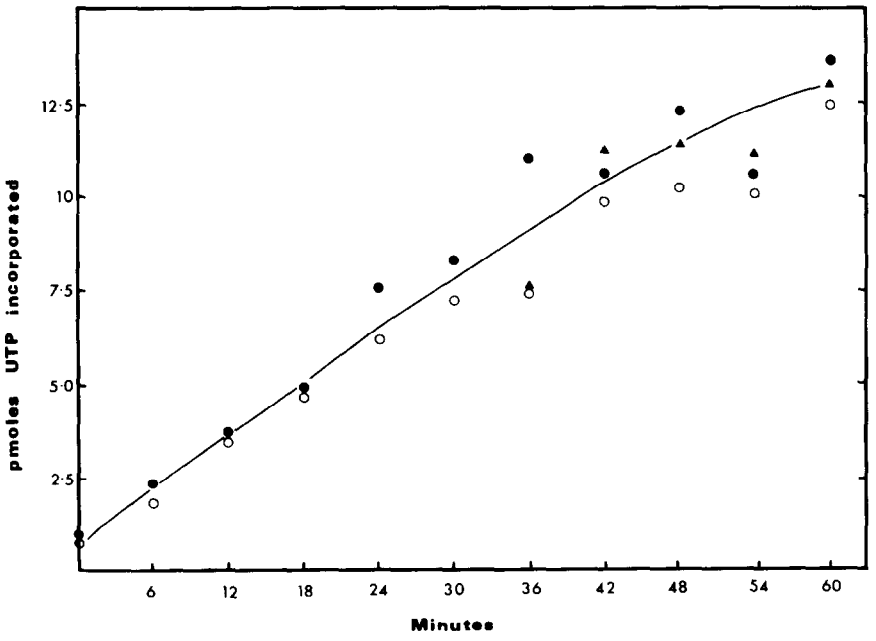

Fig. 1. Effect of α-amanitin on the release of form I RNA polymerases from nuclei. Each points shows the RNA polymerase activity of the dialysed low salt extract from nuclei derived from 3.6 g liver. ●—● 2 μg/ml α-amanitin in nuclear suspension added at zero time; ▲—▲ 2 μg/ml α-amanitin added at 30 min; ○—○, no α-amanitin.

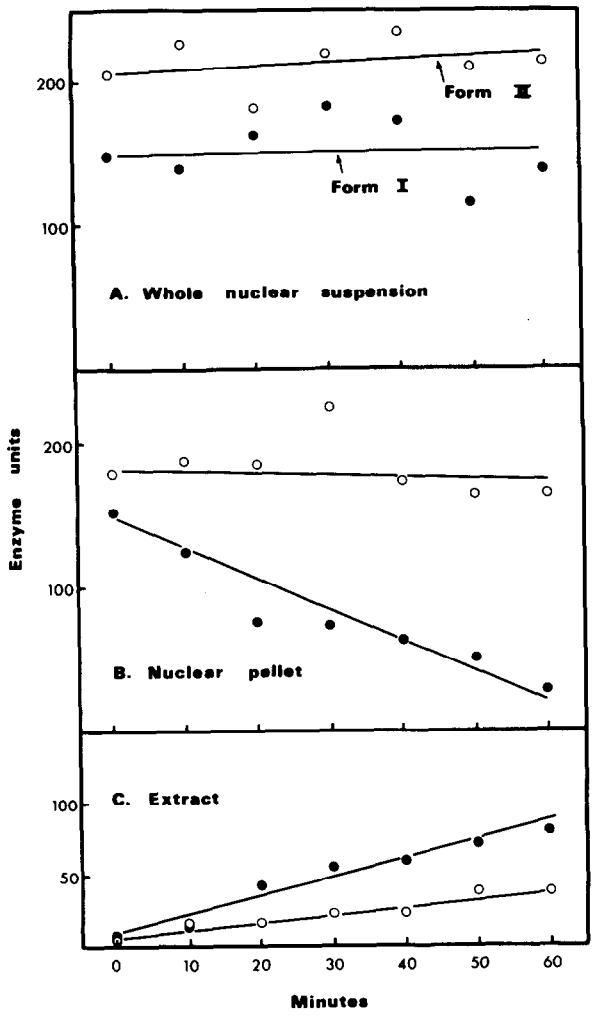

Fig. 2. Distribution of forms I and II during low salt extraction of nuclei. Each point represents a batch of nuclei derived from 14 g liver which was subjected to low salt extraction for the time indicated. Each batch was then high salt extracted or separated into nuclear pellet and extract before high salt extraction. ○—○ α-amanitin sensitive form II; ●—●, α-amanitin resistant form I.

of form I released. The results in fig. 1 show that this did not occur. Addition of α-amanitin at zero time or after 30 min had no effect on the rate of release of form I polymerases.

3.2. *Attempt to demonstrate the formation of form II from extracted form Ib*

In our model we suggest that during low salt extraction form II dissociates to yield Ib and an α-amanitin binding subunit ('A' factor). Form Ib passes out of the nucleus whilst the 'A' factor remains on the chromatin within the nucleus. However, the level of form II that can be extracted by the high salt method does not decrease during low salt incubation [2]. In these experiments the whole suspension was high salt extracted. To account for this it was suggested that form II is regenerated during high salt extraction from the extracted Ib and the 'A' factor. An experiment reported here was devised to test this idea.

Nuclei can be easily separated from the extraction buffer by light centrifugation. If regeneration of form II occurs, then such a separation of the 'A' factor within the nucleus from the form Ib in the extract should significantly decrease the level of form II subsequently extracted by the high salt method. Equal batches of nuclei suspended in the low salt extraction buffer were incubated for various periods up to 60 min at 37° and then each was split into two equal portions. One portion was directly extracted by the high salt method whilst the other was separated into extract and nuclear pellet fractions by centrifugation before high salt extraction. The recovered units of form I and II enzymes in each fraction are shown in fig. 2. As reported before [2], the levels of form I and II in the whole nuclear suspension stay fairly constant during low salt incubation. Contrary to the regeneration idea, however, the level of form II in the nuclear pellet also stays constant. The form I which appears in the extract seems to be derived from nuclear form I whose level in the nuclear pellet is depleted during low salt extraction. The origin of the Ib polymerase is therefore indicated as being form Ia or a previously inactive source.

3.3 *Efficiency of polymerase extraction*

The absolute validity of the above result depends upon a quantitative extraction technique. The recoveries of form Ia, Ib and II from nuclei are shown in table 2. Whereas low salt extraction recovers over

Table 2
Recoveries of the various forms of RNA polymerase.

Fraction	RNA polymerase (enzyme units)		
	Form Ia	Form Ib	Form II
Intranuclear		339	1321
Low salt extract	210	246	29
High salt extract	100	15	172

The results show typical yields of enzyme from nuclei derived from a 10 g batch of liver. Form I polymerases were assayed in the presence of 0.1 μg/ml α-amanitin. Form II polymerase was assayed ± α-amanitin and the sensitive activity is shown. One enzyme unit incorporates 1 pmole UTP/min into RNA.

100% of the detectable intranuclear form I activity, high salt extraction yields only 34% and 13% of the form I and II activities respectively. Such values depend upon how the intranuclear activity is assayed. Here the optimal low salt plus Mg^{2+} ions and high salt plus Mn^{2+} ions assay conditions described by Widnell and Tata were used.

4. Discussion

Clearly neither result supports the possibility of interconversion between form II and Ib. However, due to the complexity of the nuclear system and the inadequacies of current extraction methods, these results still do not exclude the occurrence of interconversion. Thus if the dissociation of form II involves the loss of an α-amanitin binding subunit and if this dissociation can still occur with α-amanitin bound, then the results of the first experiment are compatible with interconversion. Also since only 13% of the intranuclear form II activity is apparently recovered by the high salt extraction method, the findings of the second experiment can be regarded as only qualitative. It should be noted, however, that the optimal assay conditions for intranuclear and extracted form II polymerase are different, 0.4 M and 0.13 M $(NH_4)_2SO_4$ being used respectively. Using low $(NH_4)_2SO_4$ concentrations for both assays, Roeder and Rutter [4] reported a quantitative extraction of form II from rat liver nuclei with a high salt method identical to that used here. Since nothing is known about the relative activities of intranuclear and extracted enzymes, any quantitation of recoveries is open to question. Although these criticisms are important in interpreting the above experimental results, the lack of any positive evidence for interconversion leads to the conclusion that any proposed integrated nuclear transcription system must remain a matter for conjecture.

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